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SHORT COMMUNICATION

Occurrence and ecological implication of a tropical anguillid eel, *Anguilla marmorata*, in Brunei Darussalam, Borneo Island

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ABSTRACT. Tropical anguillid eels account for two-thirds of the 19 species in *Anguilla* Schrank, 1798. However, information on the species diversity, geographical distribution, and life histories of the tropical eels is very limited. Recent studies suggested that morphological species identification of the tropical anguillid eels should be validated by molecular analysis for accurate identification. After surveying for three years, two anguillid eels were found in Brunei Darussalam, Borneo Island. They were firstly identified as *Anguilla marmorata* Quoy & Gaimard, 1824 using morphological analysis and further gene analysis of cytochrome c oxidase subunit I (COI) confirmed the species identification. This study is the first comprehensive description of *A. marmorata* in Brunei Darussalam, Borneo Island. Furthermore, it is also the first study to validate two anguillid eels collected from the tropical Bonin Islands of Japan as *A. marmorata* by means of morphological and COI analyses. The molecular phylogenetic tree and haplotype network analyses suggest that *A. marmorata* found in Brunei Darussalam would belong to the North Pacific population of the westernmost distribution.

KEY WORDS. Catadromous fish, geographical distribution, giant mottled eel, tropical anguillid eel, tropical biodiversity.

The catadromous anguillid eels of *Anguilla* Schrank, 1798 are widespread throughout the world from tropical to temperate areas and consist of 19 species (Froese and Pauly 2019). Within *Anguilla*, 13 species are distributed in tropical areas and the remaining six species occur in temperate areas (Tesch 1977, Arai 2016). Molecular phylogenetic research on anguillid eels has revealed that tropical eels to be more similar to the ancestral species originating from the Indo-Pacific and that anguillid eels radiated out from the tropics to colonize the temperate regions (Minegishi et al. 2005). We expect that tropical anguillid eels resemble more to their ancestral forms than their temperate counterparts. Thus, studying the biological aspects of tropical eels could provide clues for understanding the nature of ancestral modes of catadromous migration in anguillid eels and how the large-scale migration of temperate species became established.

Comprehensive studies by Ege (1939) have discussed anguillid species diversity, geographical distribution and abundance in the world and have revealed that the highest diversity of anguillids occurs in Southeast Asian waters. However, for Brunei Darussalam, there is relatively little information available on various aspects of eel biology including species composition, distribution, life history and migration. The study of eel biology research in Brunei Darussalam could provide details on their species diversity, evolutionary pathway, and life history. Furthermore, information about the geographical distribution, species composition, and life history are not available for many tropical eels across the Indo-Pacific region.

According to a few past studies, the tropical eel species *Anguilla marmorata* Quoy & Gaimard, 1824 have been found in Brunei Darussalam (Choy and Chin 1994, Sulaiman et al. 2018). However, none of these studies performed comprehensive identification methods for the anguillid species. The identification of eels at the species level using solely visual observation is known to be difficult because of the similarities and overlapping morphological characteristics in eels, particularly tropical anguillids (Ege 1939, Watanabe et al. 2004). To validate the identification of the tropical eel species, it is crucial to utilize both morphological and molecular analyses (Arai et al. 2015, Arai and Wong 2016, Abdul Kadir et al. 2017, Wong et al. 2017).

In the present study, we conducted a survey spanning a duration of three years which resulted in the collection of two anguillid eels in Brunei Darussalam, Borneo Island. We also ex-

amined two anguillid eels from the Bonin (Ogasawara) Islands, the archipelago of subtropical and tropical islands of Japan. These eels were subjected to identification using both morphological analyses and cytochrome c oxidase subunit I (COI) gene sequence analyses. This paper describes the first confirmed record of a tropical anguillid eel, *A. marmorata*, from Brunei Darussalam and the Bonin Islands of Japan. We also discuss the ecological implication and importance of the occurrence of *A. marmorata* in these regions.

During a three-year survey between August 2016 and July 2019, two anguillid eels were caught by a fishing rod and line by local people in Temburong River (4°33'23"N; 115°10'02"E) and Tutong River (4°45'7"N; 114°40'5"E), Brunei Darussalam, northwest Borneo Island on 31 August 2018 and 19 April 2019 (Fig. 1, Table 1). Furthermore, two anguillid eels collected from the Bonin Islands (27°3'35"N; 142°12'12"E), tropical islands of Japan, on 5 and 7 June 2006 were also examined.

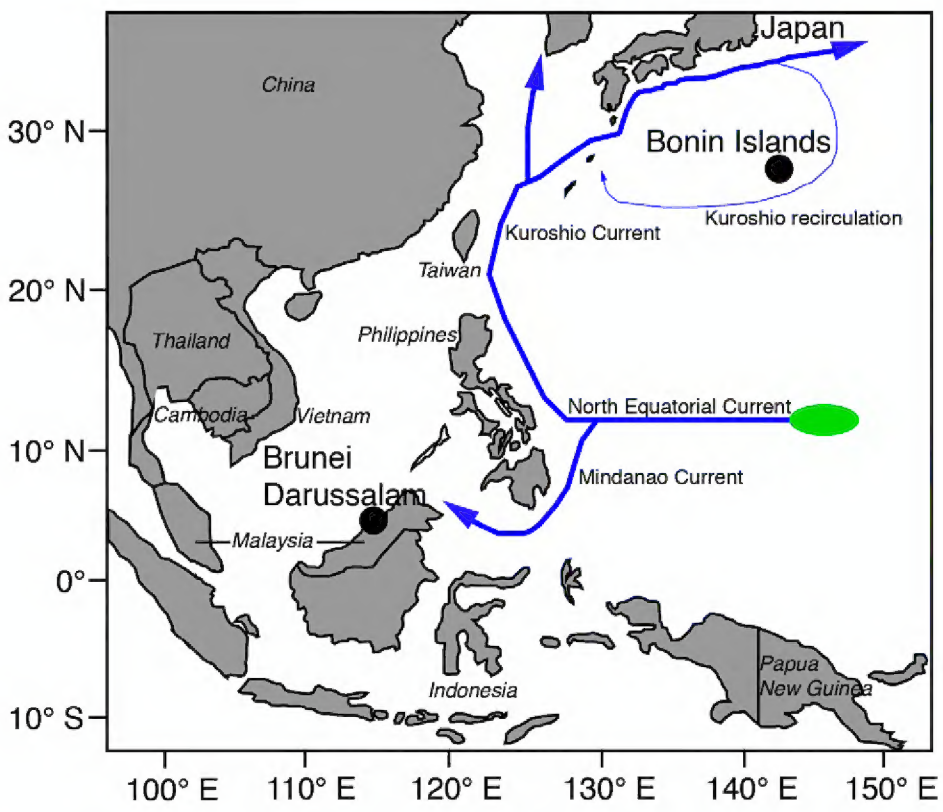


Figure 1. Map showing the collection sites of a tropical anguillid eel *Anguilla marmorata* in Brunei Darussalam, Borneo Island and in the Bonin Islands, tropical islands of Japan. The location of the offshore spawning area of *A.marmorata* in North Pacific population (green ellipse) with the oceanic currents (blue lines) from the spawning area is shown. Black circles on the map indicate the sampling location.

The external morphometric characteristics were measured following Ege (1939) and Watanabe et al. (2004), and the data are shown in Table 1. The fin difference index (FDI), which is the relative distance of the ano-dorsal length (Z) to the total length (L_T) (Ege 1939), was calculated as follows: $FDI = 100\ Z\ L_T^{-1}$. The sex of each eel was determined by visual and histological observations of the gonads. The whole gonad was weighed, and gonadosomatic index (GSI percentage of gonad weight to body weight) was subsequently calculated.

The two specimens from Brunei Darussalam were histologically examined. Tissues from the middle region of one gonad were fixed in formalin for histological analysis. Tissue fragments were prepared for resin and paraffin embedding. Paraffin blocks were sectioned at a thickness of 5 µm and stained with haematoxylin-eosin for observation. Histology classifications of female and male were according to Arai et al. (2016) and Arai and Abdul Kadir (2017). The two specimens from the Bonin Islands were not histologically examined.

The two specimens from Brunei Darussalam and two specimens from Bonin Islands were used for DNA extraction (Table 1). DNA sample was extracted from a dorsal fin clip using DNeasy Blood & Tissue Kit (QIAGEN, Germany), according to the manufacturer's instructions. Mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using different combinations of universal primers to validate the species identity, which were FishF1 (5'TCA ACC AAC CAC AAA GAC ATT GGC AC3'), FishF2 (5'TCG ACT AAT CAT AAA GAT ATC GGC AC3'), FishR1 (5'TAG ACT TCT GGG TGG CCA AAG AAT CA3'), and FishR2 (5'ACT TCA GGG TGA CCG AAG AAT CAG AA3') (Ward et al. 2009). Each PCR reaction contained 2 µl of DNA sample, 2.5 µl of each 10 µM primer, 25 µl of 2x Taq PCR Master Mix (QIAGEN, Germany) and 18 µl of distilled water. The PCR conditions were initially 95°C for 2 mins, then 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 60 s, finally 72°C for 10 mins. PCR amplicon was purified using QIAquick Gel Extraction Kit (QIAGEN, Germany), according to the manufacturer's instructions, and sequenced bi-directionally with the same primers.

Generated sequence trace files were manually edited and assembled using MEGA version 7 (Kumar et al. 2016). The contig sequences were compared for percent similarity with the reference sequences in the GenBank database (Benson et al. 2013) by using BLAST search (Altschul et al. 1990). GUIDANCE2 was used to create multiple sequence alignment via MAFFT, which also assigned the alignment with an average score of 1, indicating the robustness of the alignment (Sela et al. 2015). The multiple sequence alignment was trimmed at both ends in order to remove columns with missing data, resulting in all sequences having similar length of 506 bp with 158 polymorphic sites. No internal gap was observed in the alignment. MEGA was used to determine the best-fit nucleotide substitution model which was Hasegawa-Kishino-Yano model (HKY+G). A discrete Gamma distribution (+G) with five categories was used. MEGA was also used to construct a tree using Maximum Likelihood method. A heuristic search starting with the initial tree was conducted using Nearest-Neighbour-Interchange method with the branch swap filter set to none and the initial tree based on NJ and BioNJ algorithms. All codon positions were included in the analysis. Bootstrap test was carried out with 1000 replicates. Haplotype analysis was conducted using DnaSP version 6 (Rozas et al. 2017) and haplotype network was constructed via the reduced median method using Network version 5 (www.fluxus-engineering.com). Haplotype network was also confirmed using PopART via Integer NJ method (Leigh and Bryant 2015),

Table 1. Morphometric characters of *Anguilla marmorata* collected in Brunei Darussalam, Borneo Island (BE) and the Bonin Islands, tropical islands of Japan (OG). nd: not determined.

Measurements	BE-1	BE-2	OG-1	OG-3
Total length (mm)	962	1352	665	433
Body weight (g)	2466	8045	686	193
Gonad weight (g)	190.7	0.32	0.0	0.0
Predorsal length (PD)	282	373	178	114
Preanal length (PA)	433	587	292	189
Number of teeth of mid part of Maxillary band (NMM)	1	1	nd	nd
Fin difference index (%)	15.7	15.8	17.1	17.3
Patten of color marking of skin	variegated	variegated	variegated	variegated
Sex	female	female	undifferentiated	undifferentiated
Gonadosomatic index (%)	1.06	3.46	0.0	0.0
Maturation stage	III	V	immature	immature
Species by morphology	*A. marmorata*	*A. marmorata*	*A. marmorata*	*A. marmorata*
Species by molecular	*A. marmorata*	*A. marmorata*	*A. marmorata*	*A. marmorata*

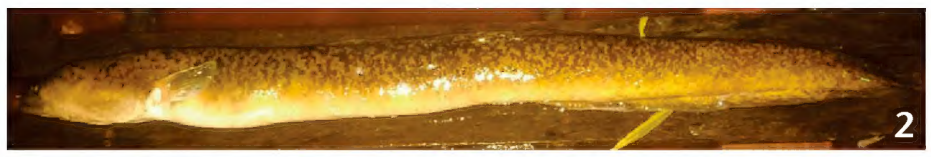
which produced similar results. Thus, only the representative result from Network is shown in this study.

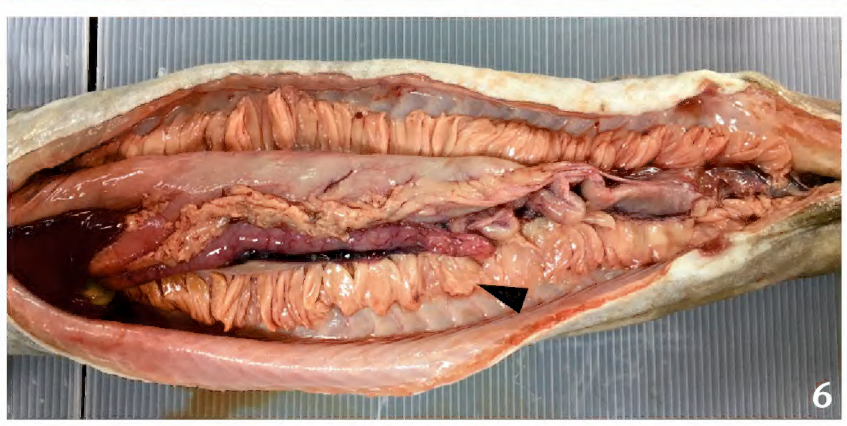
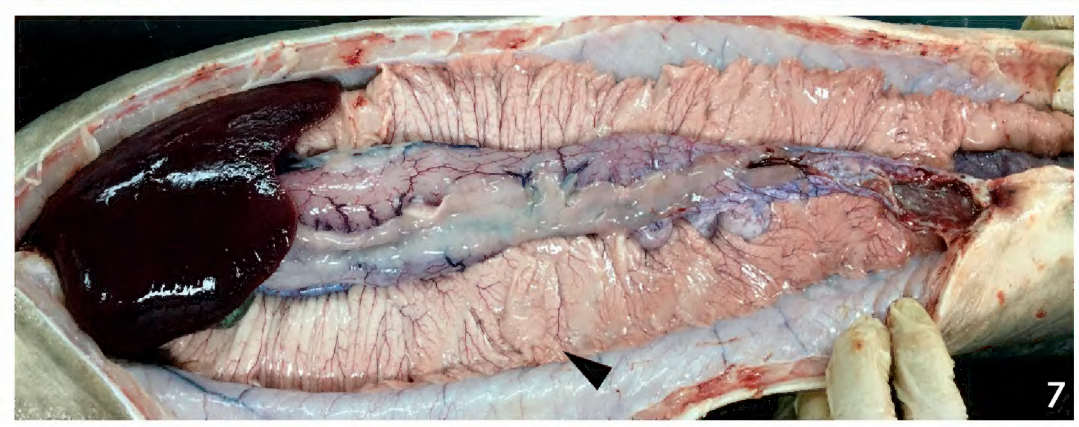
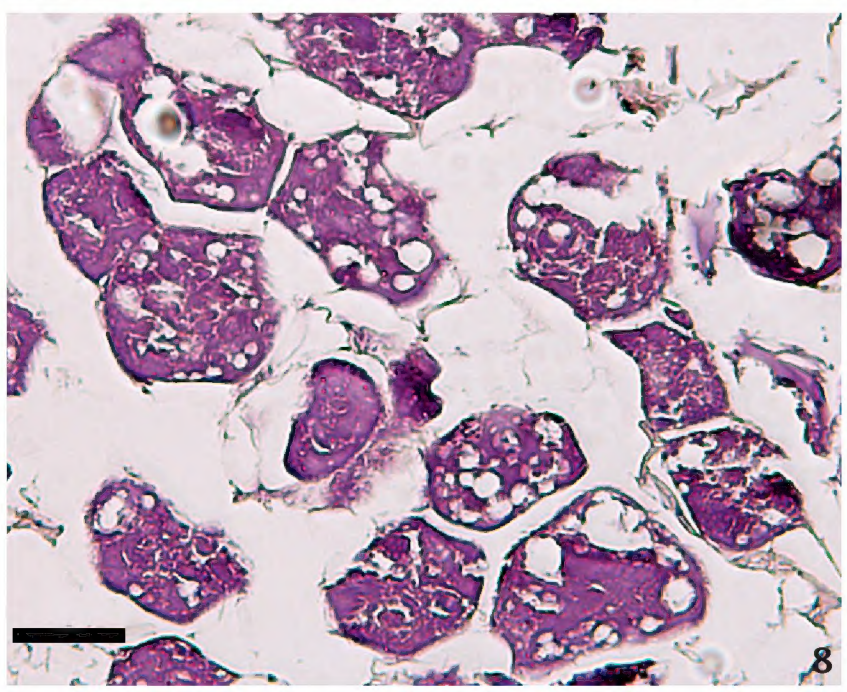
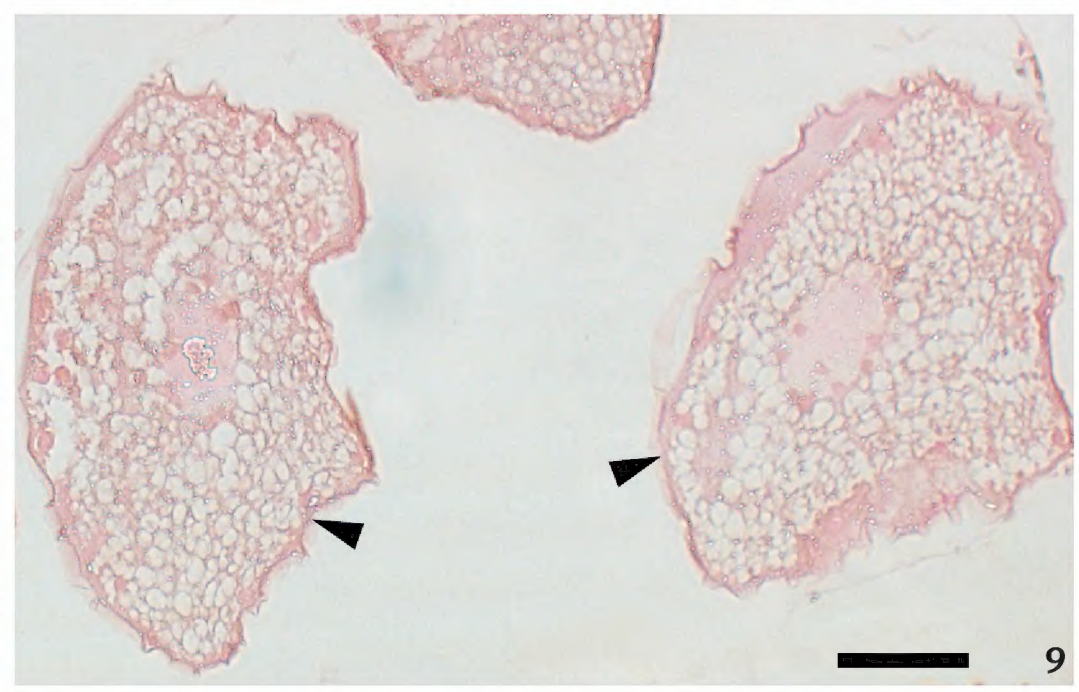
The sequences of eels from Brunei Darussalam and Bonin Islands of Japan were submitted to the GenBank database with accession numbers MN315355-MN315356 and MN315357-MN315358, respectively. In addition to these four sequences, the COI sequences of *A. marmorata* from other localities that were deposited in the GenBank database (Benson et al. 2013) were also included in the phylogenetic and haplotype analyses. The localities and GenBank accession numbers were China (Hainan HQ141374), French Polynesia (JQ431413, JQ431414), Hawaii (DQ520999, DQ5210000, Indonesia (Ache Sumatera HM345929, KY618770; Ambon AP007242; Bali KU692250-KU692249; Bengkulu Sumatera JQ665824, JQ665825; Java KU692248, KU692250-KU692252), Malaysia (Sabah MG324010-MG324012), Philippines (KC970325-KC970327), Taiwan (KU885607, KU942680, KU942730, KU942731), Thailand (MG324009) and Vietnam (MK818583-MK818585). The tree was constructed to also include the mtDNA COI sequences of other *Anguilla* species from the GenBank database together with *Serrivomer sector* Garman, 1899 as outgroup.

Four eel samples had skin with variegated markings (Figs 2, 3). Furthermore, the eels from Brunei Darussalam had narrow maxillary bands of teeth with one tooth in the mid part of maxillary band (1) (Figs 4, 5, Table 1). The four eel samples had FDI values of 15–18 % (Table 1). *Anguilla* has been clearly divided into four different species groups based on the external morphological characteristics of each species: the first group (four species) has variegated skin with broad maxillary bands of teeth, the second group (four species) has variegated skin with narrow maxillary bands of teeth, the third group (six species) has non-variegated skin with a long dorsal fin, and the fourth group (five species) has non-variegated skin with a short dorsal fin (Ege 1939, Watanabe et al. 2004, Arai 2016). The four eel samples were assigned into the second group of *Anguilla* (comprising of *A. bengalensis bengalensis* (Gray, 1831), *A. bengalensis labiata* (Peters, 1852), *A. marmorata*, *A. reindardtii* Steindachner, 1867) based on their variegated skin and narrow maxillary bands of teeth (Ege 1939, Watanabe et al. 2004, Arai 2016, Arai and Wong 2016, Abdul Kadir et al. 2017, Wong et al. 2017).

The geographical distribution of anguillids in combination with key morphological characteristics is commonly used to determine the classification of eels. Within the second group, *A. bengalensis labiata* and *A. reinhardtii* exist in the mid-southeastern region of Africa and eastern Australia and Tasmania, respectively (Ege 1939). Therefore, both of these species were not considered when species specificity of the samples was assessed in the present study. The FDI values of the other two species, *A. bengalensis bengalensis* and *A. marmorata* were considered. According to the key morphological characteristics used for identification (Ege 1939, Watanabe et al. 2004), the FDI of *A. marmorata* is in the range of 12 to 20, higher than that of *A. bengalensis bengalensis*, which is in the range of 8 to 13 (Ege 1939, Watanabe et al. 2004, Arai 2016). Based on the FDI values of 15.7 (BE-1), 15.8 (BE-2), 17.1 (OG-1) and 17.3 (OG-3), these eels were identified as *A. marmorata* (Table 1).

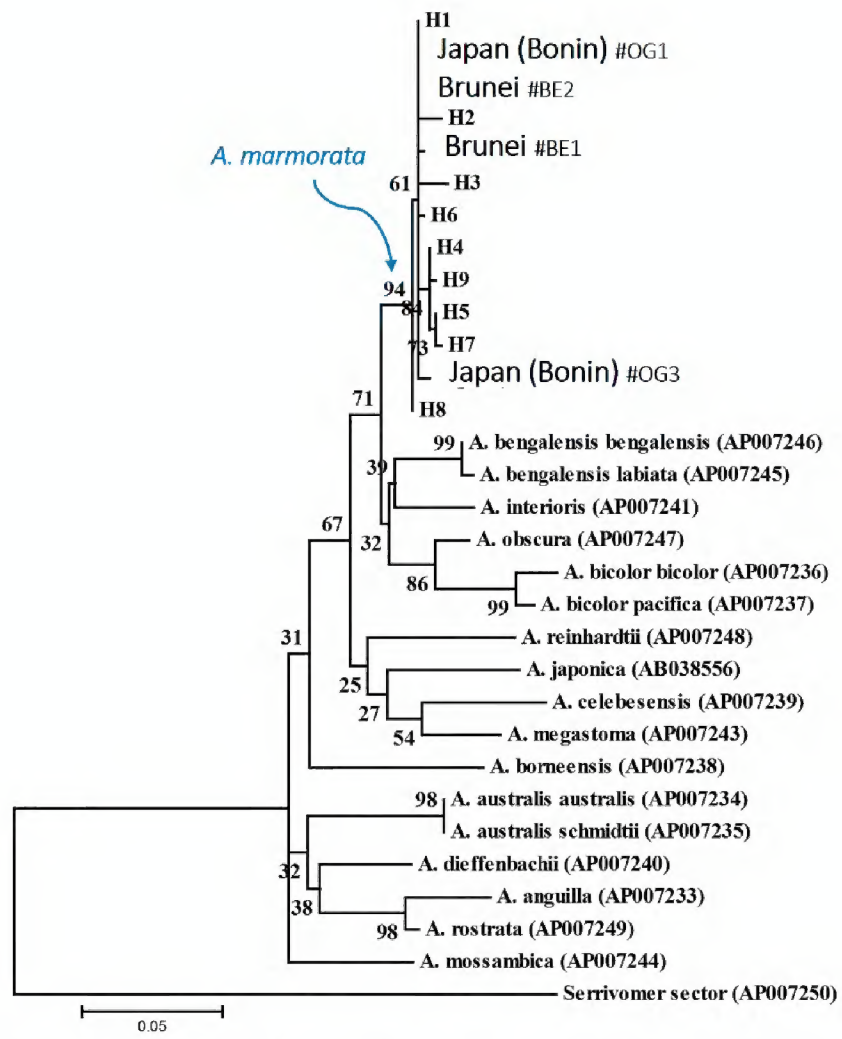
Both eels of BE-1 and BE-2 were female based on the visual observation of their gonads and their histological data (Figs 6–9, Table 1). Interestingly, the eel specimen of BE-2 had high GSI (3.46 %) and was at the final stage of maturation of stage V with mid-vitellogenic oocytes (Fig. 9, Table 1). It suggests that the eel was at a starting downstream migration to the open ocean, ready for spawning. The eel specimen of BE-1 had the GSI value of 1.06 % and the maturation stage was stage III, suggesting that the eel was at an early maturation stage with oocytes showing oil droplets (Fig. 8). Two eels from the Bonin Islands of Japan were at immature stage due to their undifferentiated sexes and without gonads (Table 1).

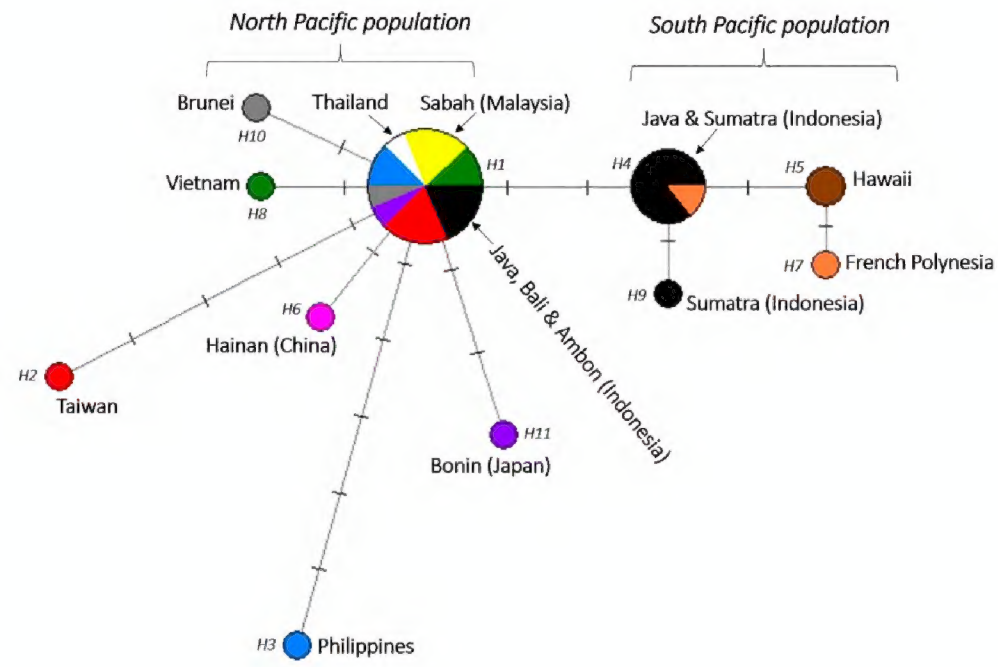


Figures 2–9. Adult specimens (2, 3) and their teeth (4, 5) and gross morphologies (6, 7) and histological sections (8, 9) of the gonads of tropical anguillid eels, *Anguilla marmorata*, that were collected in Brunei Darussalam, Borneo Island: (2) BE-1, *A. marmorata* (962 mm in TL); (3) BE-2, *A. marmorata* (1352 mm in TL); (4) Narrow maxillary bands of teeth of BE-1; (5) Narrow maxillary bands of teeth of BE-2. The gonadal histology of Stage III (8; BE-1) showed oocytes with oil droplets mid-vitellogenic oocytes in the early maturation stage. The gonadal histology of Stage V (9; BE-2) showed mid-vitellogenic oocytes (arrows) in the final preparation for spawning. Gross morphologies of the gonads (6, 7) are indicated by arrows. Scale bars: 8, 9 = 50 μm.

Molecular identification based on COI gene had confirmed that all specimens were *A. marmorata* with 99–100 % maximum identity matches with the reference sequences in the GenBank database. Haplotype analysis revealed a total of 9 haplotypes (H1 to H9) from the included 29 sequences. One eel sample from Brunei Darussalam and one eel sample from the Bonin Islands





Figures 10–11. Phylogenetic maximum likelihood tree using the mtDNA COI of *Anguilla marmorata* and other *Anguilla* species from the GenBank database with indicated accession numbers (10). *Serrivomer sector* was used as outgroup. The bootstrap proportions are shown next to the branches. Scale refers to evolutionary distance and in the unit of number of base substitutions per site. Haplotype network constructed with *Anguilla marmorata* mtDNA COI sequences (11). Each colour represents a sample site. The size of the circle is proportional to the number of samples that belong to each haplotype. Different haplotypes are labelled as H1 to H11. Each dash, which appears on the line that connects two haplotypes together, symbolizes one mutational step.

belonged to H1. Two new haplotypes (H10 and H11) were observed from the other two eel samples from Brunei Darussalam and the Bonin Islands.

A few reports have described the presence of eels in Brunei Darussalam (Choy and Chin 1994, Sulaiman et al. 2018), however these publications did not show the specific species identification. Based on the examination of morphological details, this is the first description of the occurrence and distribution of *A. marmorata* in Brunei Darussalam. However, as morphological identification could lead to misidentification, it is important for the tropical eel species identification to be accurately validated by molecular analysis as shown by the findings from previous studies (Arai et al. 2015, Arai and Wong 2016, Abdul Kadir et al. 2017, Wong et al. 2017). The species misidentification in the previous studies might have been due to an insufficient morphological characteristic analysis. In fact, the difficulty in distinguishing both *A. marmorata* and *A. bengalensis bengalensis* is augmented by their overlapping morphological characteristics, which cause further identification ambiguities (Arai and Wong 2016). Thus, a number of anguillid eels previously found in Malaysia were identified using a morphological analysis and the identification was further validated as *A. bengalensis bengalensis* by an analysis of the eels' mitochondrial COI sequences and/or 16S ribosomal RNA (16S rRNA) sequences (Arai et al. 2015, Arai and Wong 2016, Abdul Kadir et al. 2017, Wong et al. 2017). This means that comprehensive morphological analysis should be strengthened by the integration of molecular marker analysis to further validate the true identity of a species. In the present study, this is the first comprehensive description of the occurrence and distribution of *A. marmorata* in Brunei Darussalam as identified by both morphological and molecular analyses. Furthermore, this study is the first to confirm that the eels from the Bonin Islands of Japan were also *A. marmorata* by means of similar analyses.

Phylogenetic tree of *A. marmorata* and other *Anguilla* species suggests that *A. mossambica*, which inhabits southeastern Africa and Madagascar (Tesch 1977), is the most likely ancestral species (Fig. 10). The result is consistent with the molecular phylogenetic study using whole mitochondrial genome sequences (Minegishi et al. 2005). *Anguilla marmorata* formed a monophyletic group with 11 Indo-Pacific species, *A. celebesensis*, *A. interioris*, *A. megastoma*, *A. bengalensis bengalensis*, *A. bengalensis labiate*, *A. reinhardtii*, *A. borneensis*, *A. japonica*, *A. bicolor pacifica*, *A. bicolor bicolor* and *A. obscura* (Fig. 10). However, they did not form a monophyletic group with *A. mossambica* which is also an Indo-Pacific species (Fig. 10). Therefore, the biogeography

of *Anguilla* might not be simple, and the present geographic distribution could be attributed to, for example, multiple dispersal events, multidirectional dispersion, or past extinctions (Minegishi et al. 2008).

During the year-round survey for three years, we collected only two specimens of *A. marmorata* in Brunei Darussalam. This limited number of eels suggests that the region might be marginal in distribution of the species. Previous study on the population structure of the giant mottled eel, *A. marmorata*, suggested that this species has a multiple population structure as follows: (i) the North Pacific (from Japan to Sulawesi), (ii) the South Pacific (from Papua New Guinea to Tahiti), (iii) the Indian Ocean (from Sumatra to Madagascar), and (iv) Guam (including Micronesia) populations (Minegishi et al. 2008). In the present study, based on the molecular phylogenetic tree and the haplotype network analyses suggest that *A. marmorata* from Brunei Darussalam and the Bonin Islands belong to the North Pacific population (Fig. 11). The western North Pacific known to be the spawning ground of the Japanese eel *A. japonica* (Tsukamoto et al. 2011) is suggested to be a possible spawning area for *A. marmorata* of the North Pacific population (Fig. 1). *Anguilla marmorata* in Brunei Darussalam might originate from the spawning areas in the western North Pacific. However, the distance from the spawning area to the recruitment area in Brunei Darussalam is considerably longer compared to the other distribution areas including the Bonin Islands of the North Pacific population (Fig. 1), therefore the abundance of specimens that reach Brunei Darussalam might be quite low, making *A. marmorata* difficult to be discovered in the area. One specimen of *A. marmorata* (BE-2) showed the final stage of maturation and it might be about to start or had just started its downstream migration to the western North Pacific for spawning. The finding suggests that the eels from the westernmost distribution of the North Pacific population might be able to contribute the reproduction. In the Bonin Islands, previous studies have found higher eel abundance than that of Brunei Darussalam (Chino and Arai 2010, Arai and Chino 2018). *Anguilla marmorata* in the Bonin Islands might be constantly transported by means of the North Equatorial and Kuroshio currents and further transported by Kuroshio circulation (Fig. 1) from the spawning ground. Further continuous field sampling and analysis of more DNA markers should be undertaken to better understand the details of migration, distribution and life history of the tropical anguillid eels.

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